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Measurement of calcium transients in frog muscle by the use of arsenazo III

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Intracellular injection of the calcium indicator dye arsenazo III was used to measure the calcium transients occurring during depolarization of frog skeletal muscle fibres. A quantitative estimate of the rise in intracellular free calcium during a twitch was made, and the relation between membrane potential and calcium release was examined. The results also indicate that calcium in the bathing solution plays no important part in the generation of calcium transients during single twitches.

Introduction

Activation of the contractile proteins in vertebrate skeletal muscle is induced by the release of calcium ions from the sarcoplasmic reticulum (s.r.) into the sarcoplasm (see Ebashi 1976 for recent review). Direct indications of calcium transients in muscle have previously been obtained by the use of the calcium indicator dye, murexide (Jöbsis & O'Connor 1966), and the calcium-sensitive photoprotein, aequorin (Ashley & Ridgway 1970; Taylor, Rudel & Blinks 1975), but both techniques have some disadvantages. The principal difficulties with aequorin are its limited availability, and relatively slow time resolution (Hastings *et al.* 1969). Murexide does not suffer from these disadvantages, but is very insensitive.

For some years chemists have used the metallochromic dye arsenazo III (2,2′-(1,8-dihydroxy-3,6-bisulpho-2,7-naphthalene-bis (azo))-dibenzene-arsonic acid) as a very sensitive calcium indicator at neutral pH (Michaylova & Ilkova 1971), and recently this dye was used for measuring the resting calcium level (Dipolo *et al.* 1976), and the calcium influx during the action potential (Brown *et al.* 1975) in the giant axon of the squid. Basically the method involves injecting a cell with arsenazo III and monitoring changes in light absorption at a wavelength where the absorption spectrum is maximally changed in the presence of calcium. This technique permits quantitative estimates of changes in free intracellular calcium to be made (Dipolo *et al.* 1976), and gives a time resolution better than 400 μs (Brown *et al.* 1975).

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We have used arsenazo III injected iontophoretically into single fibres of frog skeletal muscle to investigate the changes in the intracellular level of ionized calcium caused by action potentials or by depolarizing pulses in voltage clamped fibres.

Methods

Preparation and electrical recording

Experiments were performed on the cutaneous pectoris muscle of *Rana temporaria*. The muscle was mounted in a glass-bottomed chamber, and stretched to a striation spacing of about 3.6 μm to reduce movement artefacts. The basic composition of the bathing solution was (in millimoles/litre) NaCl, 120; KCl, 2; $CaCl_2$, 1.8; in 1 mM sodium phosphate buffer, pH about 7.2. In experiments where fibres were voltage clamped, final concentrations of 2×10^{-6} g ml^{-1} tetrodotoxin (TTX) and 30 mM tetraethylammonium bromide (TEA) were added to the bathing solution. Unless otherwise stated experiments were carried out at 6–9 °C.

Muscle fibres were impaled with two micropipettes separated by 100–200 μm, for intracellular recording and dye injection. The dye pipette contained an aqueous solution of 1 mM arsenazo III (Sigma Chemical Co.) in 10 mM potassium bicarbonate (used to increase the solubility of the dye), brought to pH 7 with potassium hydroxide. Injection of arsenazo III was achieved by passing steady pipette-negative currents of 10–50 nA. The dye injection pipette was also used to initiate action potentials, by means of brief (2 ms) depolarizing pulses, and in some experiments to voltage-clamp a region of the fibre.

Optical recording

Measurement of changes in absorption of the arsenazo III was made by focusing light from a 50 W quartz halogen lamp on to the fibre, forming a spot of about 80 μm diameter positioned between the electrodes. The spot was viewed through a microscope fitted with a ×10 objective immersed in the bathing fluid, and a system of beam splitters placed behind the objective diverted half of the transmitted light on to two photomultipliers. Interference filters (Balzers: half-peak bandwidth 12 nm) transmitting wavelengths of 532 and 602 nm were placed in front of the photomultipliers. Before injecting dye the gains of the two photomultipliers were adjusted to give equal signals for the transmitted light, and injection of dye was then monitored by the fall in light transmission at the two wavelengths. By electronically subtracting the signals from the photomultipliers a substantial cancellation of movement artefacts could be obtained, since both channels are affected equally. On the other hand, changes in light absorption due to variations in calcium concentration summate in this record, because the absorbance of arsenazo III increases greatly in the presence of calcium at a wavelength of 602 nm, while at 532 nm the absorbance is slightly reduced.

Calibration of optical records

The output from the photomultipliers was calibrated in terms of changes in free calcium concentration by replacing the muscle with a glass capillary of 90 μm inside diameter, through which flowed solutions of arsenazo III containing known free calcium concentrations. The basic composition of the calibrating solution was (in millimoles/litre) KCl, 100; $MgCl_2$, 2; arsenazo III, 0.2; in 4 mM sodium phosphate buffer, pH 7. For solutions with free calcium levels below 1 μM, ethylene glycol bis (β-amino-ethyl-ether)-*N*,*N*-tetra-acetic acid (EGTA) was added to a final concentration of 1 mM to act as a calcium buffer. Higher calcium levels were obtained by using the arsenazo III as the calcium buffer. Corrections were made for the contaminating calcium present in the solution (about 0.1 mM). Apparent stability constants of 1.35×10^6 M^{-1} for CaEGTA (Godt 1974) and 5×10^4 M^{-1} for Ca-arsenazo III (Dipolo *et al.* 1976) were assumed in calculating the free calcium levels.

In order to standardize for differences in fibre diameters and amounts of arsenazo III injected, the photomultiplier records were expressed as the change in absorbance at the wavelength pair 532–602 nm due to calcium, divided by the absorbance of the dye at 532 nm in calcium free solution, i.e. standardized change in absorbance due to calcium $\Delta A_s = (\Delta A_{532} - \Delta A_{602})/A_{532}$.

For free calcium levels up to about 8 μM a linear relation was found between ΔA_s and [Ca^{2+}] free, with a slope of 20 μM per unit ΔA_s. At higher calcium levels the dye response began to curve off appreciably, and ΔA_s reached a limiting value of about 0.9 at free calcium levels above 30 μM.

At concentrations of arsenazo III above about 0.3 mM the main absorption peak of the dye becomes broader, and the response to calcium is diminished. These effects could be clearly seen in the photomultiplier signals during injection of a fibre with the dye, and records were generally obtained at a dye concentration a little below that required to produce a maximum calcium response.

Results

Calcium changes caused by single action potentials

The changes in light absorption which accompany an action potential in a fibre injected with arsenazo III are illustrated in figure 1. A large increase in light absorption was seen at the wavelength pair 532–602 nm (middle trace), where the dye is maximally sensitive to changes in calcium concentration. The upper trace shows the change in absorbance at 532 nm, where the dye is relatively insensitive to calcium, and demonstrates that movement of the fibre did not give rise to any serious artefacts. The deflexion below the baseline on this trace was produced by contraction of the fibre, but would have been almost completely cancelled out on the 532–602 nm trace. Usually, no deflexion of the 532–602 nm trace was seen in response to action potentials before injection of arsenazo III, indicating that this recording responds only to changes in the absorption spectrum of the dye.

Arsenazo III is not an entirely specific indicator for calcium, but the only other ion of biological importance to which it responds is magnesium, and the sensitivity to this ion is very low. An increase in magnesium concentration of about 3 mM would be required to produce the absorbance change shown. It is also unlikely that changes in pH of the muscle fibre gave rise to the dye response. It has been

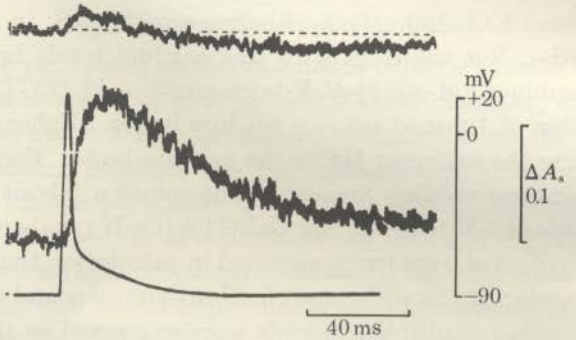

FIGURE 1. Light absorbance records from a fibre injected with arsenazo III, showing the response to an action potential. Upper and middle traces show respectively the absorbance changes at 532 nm, and at the wavelength pair 532–602 nm. The lower trace is the membrane potential. The absorbance traces were recorded through a low pass filter of time constant 0·5 ms, and the calibration (ΔA_s) gives the change in absorbance at the given wavelengths divided by the total absorbance of the injected dye at 532 nm. A just suprathreshold depolarizing pulse of 2 ms duration was used to initiate the action potential. Temperature, 7.5 °C.

demonstrated that the intracellular pH of muscle fibres becomes more acid during a twitch (Macdonald & Jöbsis 1976), and alteration in pH from 7 to 6.5 has virtually no effect on the absorption of the dye, or on its sensitivity to calcium. For these reasons we believe that the absorption record at 532–602 nm reflects mainly changes in intracellular calcium concentration.

At a temperature of 7 °C the myoplasmic calcium concentration begins to rise shortly after the peak of the action potential, and reaches a maximum after a further 15–20 ms. The subsequent fall in concentration occurs exponentially, with a time constant of about 50 ms.

The maximum change in light absorption at 532–602 nm measured in forty-nine fibres over a period between June and November 1976 was $\Delta A_s = 0.26 \pm 0.15$ (mean $\pm$ 1 s.d.), which corresponds to an increase in free myoplasmic calcium concentration of 5.25 μM, using the calibration factor given in the methods. Possible sources of error in this value could arise if arsenazo III reduced the free calcium concentration by chelating significant amounts of calcium, or had some toxic effect on the fibre. In fact the calcium in the myoplasm appeared to be well buffered, since during injection of the dye the estimated increase in free calcium from the action potential remained practically constant over a fourfold range of dye concentrations. This observation also suggests that arsenazo III does not have any significant effect on calcium release from the s.r.

Onset of the calcium response

There is a definite latency between the rise in membrane potential and the onset of the calcium change (figure 2*a*). By measuring from the foot of the action potential (about -40 mV), which corresponds closely with the threshold for calcium release (cf. figure 4), a delay of 1.8 ± 0.5 ms was found in twenty fibres.

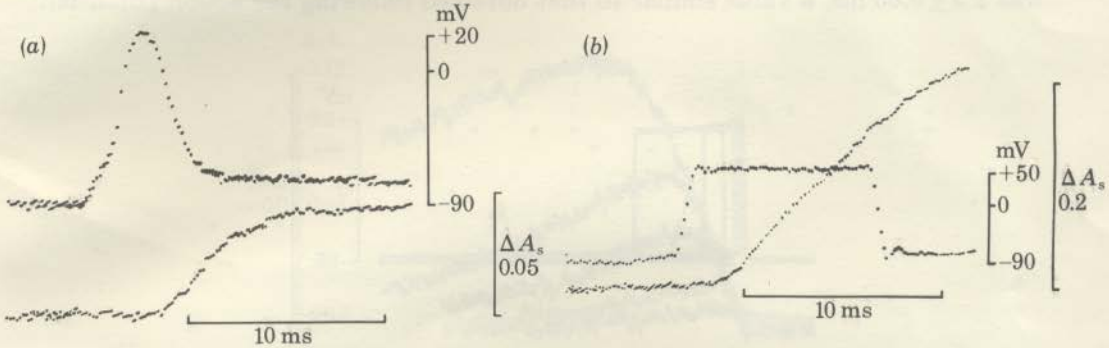

FIGURE 2. Increase in sarcoplasmic calcium following an action potential (*a*), and a depolarizing pulse (*b*). In both figures the upper trace is the membrane potential, and the lower trace shows the change in light absorbance at the wavelength pair 532–602 nm. The recording time constant for the absorbance records was 0.1 ms. Sixty sweeps were averaged in *a*, and twenty in *b*. Stimuli were given at 10 s intervals in both cases. Temperature, 5 °C in both *a* and *b*.

The reaction time of the dye is faster than 400 μs (Brown *et al.* 1975), and therefore cannot account for the delay. The temperature dependence of the delay is high (mean $Q_{10} = 3.2$ between 5 and 15 °C), suggesting that it originates from some step in the calcium release mechanism, and is not attributable to diffusion delays within the myoplasm.

Glycerolated fibres

A number of experiments were performed on muscles which had been treated with 0.4 M glycerol for 12 h, and then returned to normal Ringer after an additional period of 2 h in Ringer solution containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$; a procedure which disrupts the *T* system and blocks excitation–contraction coupling, while leaving the action potential unaffected (Fujino, Yamaguchi & Suzuki 1961; Eisenberg & Eisenberg 1968). After such treatment we were unable to see any calcium response, not even following trains of action potentials or during strong depolarizations lasting for up to one second.

Membrane depolarization and calcium release

To examine the relation between membrane potential and calcium release, a voltage clamp was used to control the membrane potential at the measuring spot.

The performance of the clamp was improved by the addition of TTX (2×10^{-6} g/ml) and TEA (30 mM) (final concentrations) to the bathing medium. Neither agent appeared to alter the calcium responses.

Figure 2*b* shows the rise in sarcoplasmic calcium concentration produced by a 10 ms depolarizing pulse to +50 mV. The calcium concentration begins to rise shortly after the start of the pulse, and continues to rise linearly throughout the duration of the depolarization. In nine fibres the latency of the calcium response was 2.3 ± 0.65 ms, a value similar to that obtained following the action potential.

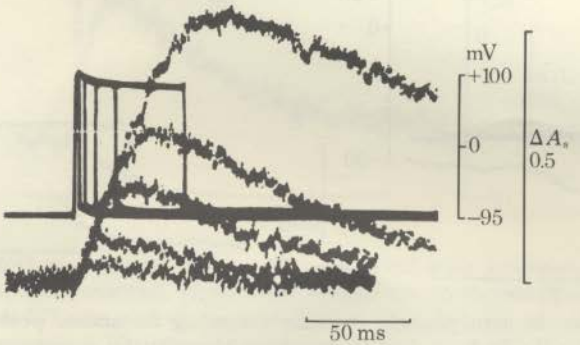

FIGURE 3. Calcium responses evoked by depolarizing pulses of different durations. Upper trace is the membrane potential, and the lower trace shows the change in light absorption at 532–602 nm. Superimposed records show pulses with durations of 2, 5, 10, 20 and 50 ms. Temperature, 6.5 °C.

If the membrane is depolarized to a potential above that required to give a maximum calcium release (cf. figure 4), for pulse durations up to about 20 ms the calcium concentration rises linearly during the pulse, but with longer durations the response begins to round off (figure 3). Some of the rounding off may be accounted for by reuptake of calcium, and the calcium concentrations involved are close to those at which arsenazo III becomes saturated. All this makes it difficult to determine whether the rate of calcium release is reduced after the first 20 ms. The mean rate of increase in ΔA_s for supra-maximal pulses shorter than 20 ms, measured in four fibres, was 0.047 ± 0.01 ms^{-1}, which gives an estimated rate of increase in free calcium concentration of 0.95 μM ms^{-1}. The decline in calcium concentration after the pulses becomes slower with large calcium responses (above about 10 μM), and is linear, rather than exponential, suggesting that the reuptake mechanism is saturated.

Figure 4 shows results from one experiment in which depolarizing pulses of 10 ms duration were used to explore the relation between membrane potential and calcium release. In this fibre the threshold potential for a just detectable calcium response was −40 mV, and a maximum response was obtained at potentials above about +10 mV. A variation in threshold potential between −35 and −50 mV was seen in nine other fibres studied. Altering the duration of the depolarizing pulse (between 5 and 40 ms), or the holding potential of the fibre (between −60 and

−100 mV), had no effect on the general form of the relation between membrane depolarization and calcium response.

During depolarization of squid nerve terminals it has been possible to prevent the entry of calcium into the axon by making the inside sufficiently positive (Katz & Miledi 1967). A similar procedure was used here to examine the possible rôle of external calcium in the release of calcium from s.r. (Ford & Podolsky 1972; Chiarandini & Stefani 1973; Stefani & Chiarandini 1973). By making the inside of

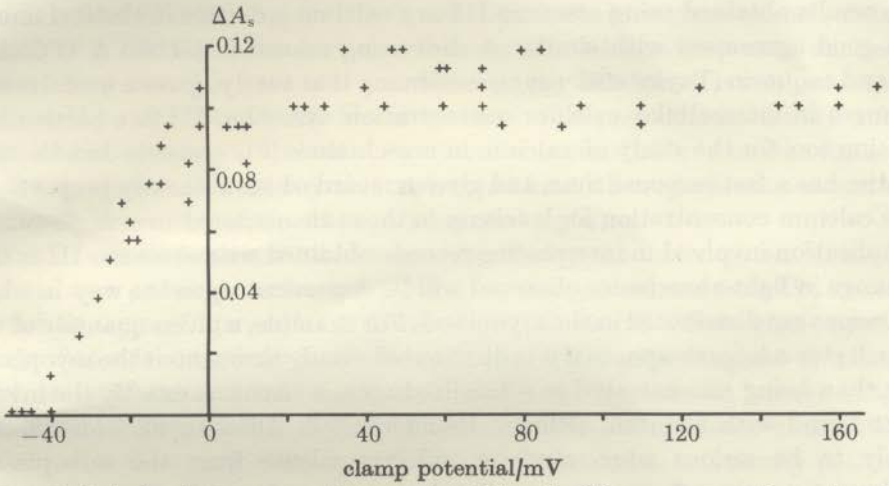

FIGURE 4. Relation between membrane potential and calcium response for a depolarizing pulse of 10 ms duration. The membrane potential was held at −75 mV between test pulses. Temperature, 7 °C.

the fibre sufficiently positive the driving force for calcium entry should be reduced, and the highest potential used in figure 4 (+170 mV) should have prevented any calcium entry, even if the internal free calcium concentration were as low as 10^{-9} M. The observed calcium response remained constant over the potential range +20 to +170 mV, suggesting that the entry of external calcium plays no important part in calcium release from the s.r. during single twitches.

Reduction of external calcium concentration

The effects of reducing the calcium in the external medium to very low levels was studied by replacing the normal solution by a solution of the following composition (in millimoles/litre): NaCl, 120; KCl, 2.5; $MgCl_2$, 5; EGTA, 1; in 1 mM phosphate buffer, pH 7. The free calcium concentration in this solution would have been less than 10^{-7} M, assuming the total concentration of contaminating calcium to be 0.1 mM.

Action potentials could be elicited for several hours after changing to the low calcium solution, although the resting potentials fell by about 20 mV. The calcium response induced by an action potential was reduced by about 30% but this

effect appears to have been secondary to a change in the action potential, since the calcium response produced by depolarizing pulses (10 ms duration) was not found to be significantly altered. The threshold potential for calcium release, and the relation between membrane potential and calcium response were the same in both normal and low calcium solutions.

Discussion

The results obtained using arsenazo III as a calcium indicator in skeletal muscle are in good agreement with similar studies using murexide (Jöbsis & O'Connor 1966) and aequorin (Taylor *et al.* 1975), confirming that the dye gives a useful record of changes in intracellular calcium concentration. Arsenazo III is a particularly promising tool for the study of calcium in muscle since it is easier to handle than aequorin, has a fast response time, and gives a record which is linearly proportional to free calcium concentration for levels up to those encountered in a single twitch. A complication involved in interpreting records obtained with arsenazo III is that the change in light absorbance observed will be dependent upon the way in which calcium ions are distributed in the myoplasm. For example, a given quantity of calcium will give a larger response if it is distributed evenly throughout the myoplasm, rather than being concentrated in a localized area, a situation exactly the inverse of that found with aequorin (Blinks, Prendergast & Allen 1976). This effect is unlikely to be serious when studying calcium release from the sarcoplasmic reticulum, since the release sites are distributed throughout the fibre at spacings of a few micrometres. The main disadvantages of the arsenazo III technique are the necessity to abolish contraction (e.g. by stretching or hypertonic solution) in order to avoid movement artefacts, and the possibility of saturation effects occurring during tetani or prolonged depolarization.

The value of 5.25 μM obtained for the peak calcium concentration during a twitch is within the range expected from work on the activation of contraction in skinned muscle fibres, where half maximum activation has been reported at free calcium levels between 0.6 and 32 μM (Ebashi & Endo 1968; Hellam & Podolsky 1969; Godt 1974). Uncertainties in the values of stability constants for CaEGTA and Ca-arsenazo III probably constitute an important source of error in our measurement, and an additional allowance should be made for the fact that the muscles were stretched to a striation spacing of 3.6 μm. Taylor *et al.* (1975) have shown that under these conditions the calcium transient is reduced to about one-third of that at the resting length. The accuracy of the measurement also, of course, depends upon the assumption that the sensitivity of arsenazo III inside the muscle fibre is the same as in our calibrating solution.

Calcium release from the s.r. was found to vary as a sigmoid function of membrane potential over a range of about -40 to $+10$ mV in fibres treated with TTX and TEA (figure 4). The existence of a radial voltage gradient along the *T* tubules cannot account for more than a small part of this gradation (Adrian, Costantin &

Peachey 1969), and it is likely that the observed relation reflects primarily the properties of the calcium release mechanism. The smooth gradation in calcium response with membrane potential indicates that the release of calcium from the s.r. is unlikely to be a regenerative process, and suggests that the 'calcium releasing calcium' phenomenon observed in skinned fibre preparations (Endo, Tanaka & Ogawa 1970; Ford & Podolsky 1972) is not an important mechanism under physiological conditions. In agreement with earlier studies (Heistracher & Hunt 1969; Adrian, Chandler & Hodgkin 1969), we find that sodium activation and delayed rectification appear to play no direct rôle in calcium release, since the addition of TTX and TEA had no effect on the calcium responses elicited by depolarizing pulses. We were also unable to see any effect on the calcium response which could be attributed to the blocking of active propagation down the *T* tubules (Gonzalez-Serratos 1971; Costantin 1970), but this could simply be due to the fact that the shortest pulses employed (5 ms) would have allowed effective electrotonic spread into the centre of the fibre (Adrian *et al.* 1969).

Influx of external calcium into muscle fibre during the action potential has been suggested as a possible mechanism controlling calcium release from the s.r. (Ford & Podolsky, 1972; Chiarandini & Stefani 1973; Stefani & Chiarandini 1973). Our findings that calcium release is unaffected under conditions designed to reduce or abolish entry of external calcium do not support this hypothesis, but are in agreement with earlier experiments which showed that contractions could still be obtained after the inside of the muscle fibre had been made sufficiently positive to prevent external calcium from entering the fibre (R. Miledi, unpublished data), and with experiments which showed that twitches are observed in muscles bathed in solutions containing very low calcium levels (Armstrong, Bezanilla & Horowicz 1972).

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